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Tumorigenicity by Human Papillomavirus Type 16 E6 and E7 in Transgenic Mice Correlates with Alterations in Epithelial Cell Growth and Differentiation

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The human papillomavirus type 16 (HPV-16) E6 and E7 oncogenes are thought to play a role in the development of most human cervical cancers. These E6 and E7 oncoproteins affect cell growth control at least in part through their association with and inactivation of the cellular tumor suppressor gene products, p53 and Rb. To study the biological activities of the HPV-16 E6 and E7 genes in epithelial cells in vivo, transgenic mice were generated in which expression of E6 and E7 was targeted to the ocular lens. Expression of the transgenes correlated with bilateral microphthalmia and cataracts (100% penetrance) resulting from an efficient impairment of lens fiber cell differentiation and coincident induction of cell proliferation. Lens tumors formed in 40% of adult mice from the mouse lineage with the highest level of E6 and E7 expression. Additionally, when lens cells from neonatal transgenic animals were placed in tissue culture, immortalized cell populations grew out and acquired a tumorigenic phenotype with continuous passage. These observations indicate that genetic changes in addition to the transgenes are likely necessary for tumor formation. These transgenic mice and cell lines provide the basis for further studies into the mechanism of action of E6 and E7 in eliciting the observed pathology and into the genetic alterations required for HPV-16-associated tumor progression.

Cellular growth and differentiation must be precisely regulated in order to promote normal development and to maintain tissue homeostasis. This regulation is achieved through responses by the cell to intracellular and extracellular growth signals. Studies on retroviruses led to the identification of a class of genes, oncogenes, that promote cell growth and tumorigenesis. More recently, genetic analysis of human cancers led to the identification of a second class of genes, tumor suppressor genes, which suppress cell growth and tumorigenicity. These two classes of genes are believed to be important in mediating the cell's response to growth signals. Thus, disturbances in the balanced response to these signals can result from alteration of oncogenes or tumor suppressor genes, causing abnormal cell growth and differentiation and contributing to carcinogenesis. Such imbalances have been experimentally induced in vivo through the generation of transgenic mice (for reviews, see references 1 and 18). In several such studies, aberrant expression of cellular proto-oncogenes or expression of mutationally activated proto-oncogenes such as those originally isolated from retroviruses results in abnormal cell proliferation and in tumorigenesis. Oncoproteins from the DNA tumor viruses, simian virus 40 (SV40), polyomavirus, and bovine papillomavirus also have been shown to be inducers of carcinogenesis in transgenic mice. Early events in the course of disease in these mice often involve the inhibition of cellular differentiation and concomitant induction of abnormal cellular proliferation. In this transgenic mouse study we describe the in vivo properties of E6 and E7, the two oncoproteins encoded by human papillomaviruses (HPV) that have been implicated in cervical cancer (for a review, see reference 63).

The E6 and E7 gene products belong to a family of oncoproteins that appear to affect cell growth and differentiation at least in part through their interactions with cellular tumor suppressor genes. The E7 protein, like E1A and the large tumor antigens (Tag) of SV40 and polyomaviruses, efficiently associates with the retinoblastoma susceptibility gene product, Rb (13). Among the different HPV genotypes, the E7 proteins encoded by those HPVs highly associated with cervical carcinoma (HPV type 16 [HPV-16] and HPV-18) are most efficient in binding Rb (15, 41). The interaction of E7 with Rb is thought to affect Rb's activities, including its capacity to bind the cellular E2F transcription factor (47). The E6 protein, like SV40 Tag and the adenovirus 55-kDa E1b gene product, is capable of binding the p53 protein (60), now understood to be a tumor suppressor gene (for a review, see reference 32). The binding of E6 to p53 leads to an increased instability of the p53 protein (53). Tissue culture studies have demonstrated that HPVs associated with cervical carcinoma, specifically the viral E6 and E7 genes, exhibit immortalizing properties in human foreskin keratinocytes (19, 21, 61) and alter keratinocyte differentiation (21, 40, 61). The E6 and E7 genes are not sufficient to induce tumorigenicity but are able to cooperate with an activated *ras* oncogene to transform baby rat kidney cells (36, 49, 56). Genetic analyses indicate that E7's capacity to bind Rb is necessary for its oncogenic activity in many, though not all, cell types (14, 23, 48, 59). Furthermore, whereas the Rb and p53 genes are mutationally inactivated in HPV-negative cervical carcinomas, they are wild type in primary, HPV-positive cervical carcinomas (9, 52). This provides indirect evidence for the biological importance of E6 and E7 interactions with p53 and Rb. In sum, the observations made to date indicate that E6 and E7 may be potent *trans*-dominant negative effectors of tumor suppressor protein function.

The identified cellular targets of E6 and E7, p53 and Rb, are thought to be key intracellular regulators of cell growth

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and differentiation. Rb protein, whose genetic disruption is a hallmark of retinoblastoma, is a nuclear protein whose phosphorylation state and ability to associate with a cellular transcription factor, E2F, appear to be regulated during the cell cycle (for a review, see reference 55). The expression of Rb and the phosphorylation state of the Rb protein are found to be altered during cellular differentiation (6, 57). These observations have raised the possibility that Rb protein is an important modulator of cell cycle and cell differentiation. It is also thought that p53 is a regulator of cell cycle and/or cell differentiation (see reference 11 and references therein). This protein has recently been identified to be a DNA sequence-specific transcriptional transactivator (25), and inactivation of this activity correlates with cellular transformation (62). In mice, disruption of both alleles of the p53 gene did not lead to any apparent developmental defects, although these mice, as adults, did develop tumors with rapidity (11). Targeted expression of the E6 and E7 proteins in transgenic mice, given their capacity to inactivate p53 and Rb function, could provide a powerful means to address the function of these tumor suppressor genes in cell growth and differentiation and in tumorigenesis.

To define in vivo the biological activities of E6 and E7, we have generated transgenic mice in which we have directed expression of these viral genes to the mouse ocular lens. The lens is an epithelial tissue which undergoes a well-characterized two-stage process of differentiation during embryogenesis (50). Like the epidermis, which is the natural target tissue for the papillomaviruses, the lens is composed of a single layer of undifferentiated cells, with the remaining layers consisting of cells in various stages of differentiation. Cells in a restricted region of the undifferentiated layer of the lens have the capacity to proliferate throughout life, thereby renewing the population of cells which then undergo differentiation. Expression of a transgene can be targeted to the lens by using the lens-specific αA crystallin promoter (43). Because the αA crystallin promoter is active in the lens from early in its embryonic development through adulthood, the long-term effects of the transgene on epithelial cell growth, differentiation, and tumorigenesis can be assessed. This approach has been previously used to study the tumorigenic capacities of oncogenes from other DNA tumor viruses (16, 35). Targeting expression to the lens offers the advantage that this tissue is not essential for the viability of the animal, thus avoiding the potential for lethality associated with disrupting cellular growth and differentiation in some developing tissues. Our present studies indicate that HPV-16 E6 and E7 alter the growth and differentiation of epithelial cells and can lead to the high incidence of tumor formation.

MATERIALS AND METHODS

Transgene construction and generation of transgenic lineages. The HPV-16 E6 and E7 open reading frames were amplified by polymerase chain (PCR) with a full-length clone of HPV-16 DNA as a template. The *Bam*HI-tailed oligonucleotides oligo 1 and oligo 4, corresponding to HPV-16 sequences from nucleotide (nt) 79 to 101 and from nt 883 to 864, respectively, were used. The amplified fragment was cloned into the vector αIpA at the unique *Bam*HI site. This cloning inserts the HPV-16 E6 and E7 sequences downstream from the murine αA crystallin promoter and upstream from the SV40 polyadenylation sequences in the bacterial plasmid vector, pUC18. The transcriptional start site is within the αA crystallin promoter, and the translation initiation sites are within the E6/E7 sequences. The construct was sequenced to verify the integrity of the E6 and E7 open reading frames and the junction of the αA crystallin promoter to the E6/E7 sequences.

For microinjection, the 1.45-kb fragment, which contains the promoter, HPV-16 E6 and E7 genes, and SV40 polyadenylation signals, was isolated by digestion with *Xma*I and *Sal*I restriction enzymes, which cleave once each within the pUC18 polylinker, and purified by agarose gel electrophoresis as described previously (16). The purified DNA fragment was microinjected into the male pronuclei of one-cell fertilized embryos (FVB/N × FVB/N) as described (16, 20) by the University of Wisconsin Biotechnology Center's Transgenic Mouse Facility. The transgene was detected by Southern blot analyses of genomic DNA from tail biopsies of 3-week-old mice (16). The radiolabeled HPV-16 E6 and E7 DNA probe was made by using the random primed labeling method. The 800-bp E6 and E7 DNA used as probe was produced by PCR amplification of sequences from the full-length clone of HPV-16 DNA, by using oligo 1 and oligo 4 as primers.

Analysis of E6 and E7 expression by RNA PCR. Tissues were excised, lenses were dissected from the eye, and material was frozen immediately on dry ice. Tissues except for lens were pulverized on dry ice and scraped into guanidine isothiocyanate for RNA purification. For lenses, tissue was ground with a sterile guanidine isothiocyanate as the tissue thawed. Total RNA was prepared by the procedure of Chirgwin et al. (7). RNA was treated with RNase-free DNase to remove any residual DNA contamination and quantified by UV absorption, and its integrity was evaluated on ethidium bromide-stained gels. For E6 and E7 expression analysis, RNA PCR was performed on 2.5 μg of total cellular RNA, as previously described (38), by using oligonucleotide primer pairs, oligo αP1-oligo 2, oligo αP1-oligo 4, or oligo 3-oligo 4, as indicated in the text. The primer oligo αP1 corresponds to the sequence of murine αA crystallin gene from nt 4 to 25 (equivalent to the sequence from nt 371 to 395 [26]). oligo 2 is complementary to the HPV-16 sequence from nt 559 to 539, and oligo 3 is identical to the HPV-16 sequence from nt 554 to 578. oligos 1 and 4 are described above. For brain, intestine, liver, tail, and eye samples, amplification of the endogenous mouse urokinase transcript, which is a gene known to be expressed in these tissues (38), was also performed as a control for integrity and quality of the RNA preparation. For RNA PCR of the urokinase transcript, the primer upa17 was identical to the sequence of the mouse urokinase gene from nt 11 to 35, and the primer upa10 was complementary to the sequence from nt 480 to 456 (4). For lens samples, which do not express urokinase, amplification of αA crystallin transcript was performed as a control by using the primers αP1, described above, and α1, which is complementary to the murine αA crystallin sequence from nt 590 to 565 (26). At the conclusion of PCR, samples were digested with RNase A for 10 min at room temperature. The PCR products were then resolved on 2% agarose gels and either visualized by ethidium bromide staining or transferred to nitrocellulose for Southern hybridization to randomly primed, labeled [α-^{32}P]HPV-16 E6 and E7 DNA.

Quantitative PCR analysis was performed according to the procedure of Wang and Mark (58). A 2.5-μg amount of total lens RNA from neonatal line 4, 18, and 19 lenses was reverse transcribed with oligo 4. Serial dilutions of the cDNA product (corresponding to 2.5 to 100 ng of the original input RNA) were then PCR amplified for 25 cycles by using oligo 3 and oligo 4, in which oligo 4 was previously kinased by using [γ-^{32}P]ATP. The ^{32}P-radiolabeled PCR products were

then resolved by polyacrylamide gel electrophoresis (PAGE), the dried gel was exposed to Kodak XAR5 film without a screen, and the amount of radioactive product was quantified by densitometric scanning of the autoradiograph. For generation of standards for quantification of product, E7-specific RNA was transcribed in vitro by using SP6 polymerase, the amount of SP6 RNA product was quantified on agarose gels, cDNA was then generated by reverse transcription of a specific amount of SP6 RNA by using oligo 4 as indicated above, and serial dilutions of the cDNA (corresponding to 0.3×10^6 to 2.4×10^6 molecules of E7-specific RNA) was PCR amplified as indicated above. PCR mixtures were run in quadruplicate for each serial dilution of standards, and lens samples within an experiment and the experiments were repeated three times each.

Histological analysis of the eye. Eyes were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 16 to 24 h at 4°C, dehydrated in increasing concentrations of ethanol, and embedded in methacrylate (JB-4 embedding medium; Polysciences, Inc. Warrington, Pa.). Sections (1.5 μm), vertically oriented, parallel to the optic axis, were cut on an ultramicrotome. Sections were then stained with hematoxylin-eosin and examined by light microscopy. Embryos were staged by designating midday on the day of the vaginal plug as day 0.5 of development.

Establishment of cell lines from neonatal lenses. Lenses were microdissected from neonatal eyes of nontransgenic FVB/N, αAHPV-16E6/E7 line 4 and 19 heterozygous mice. Cells were established in culture as previously described for nontransgenic and αAPyLT transgenic lenses (16, 17). For continuous culture, cells were maintained in Dulbecco's modified Eagle medium–10% fetal bovine serum–100 U of penicillin ml^{-1}–100 mg of streptomycin ml^{-1}–100 U of nystatin ml^{-1} and passaged 1:10 at confluence with trypsin-EDTA.

Tumorigenicity assays. For soft agar assays, 10^5 cells from the cell passages indicated in the text were seeded in medium containing 0.35% Noble agar as described by Lambert and Howley (29). After 4 weeks in culture, the numbers of colonies exceeding 0.1 mm in diameter were counted.

For tumorigenicity assays, 10^7 cells per 75 μl of Dulbecco's modified Eagle medium or minced tumor tissue were injected subcutaneously into αAHPV-16E6/E7 syngeneic neonates. When tumors exceeded 1 cm in diameter, mice were euthanized and tumors were excised and analyzed by histology. For establishment of cell lines from the tumors, tissue was washed in PBS, minced, trypsinized for 15 to 30 min at room temperature and placed in culture dishes with Dulbecco's modified Eagle medium–10% FBS–100 U of penicillin ml^{-1}–100 mg of streptomycin ml^{-1}–100 U of nystatin ml^{-1}. After 24 h, nonadherent tissue was removed, monolayers were washed, and attached cells were allowed to grow to establish lines.

RESULTS

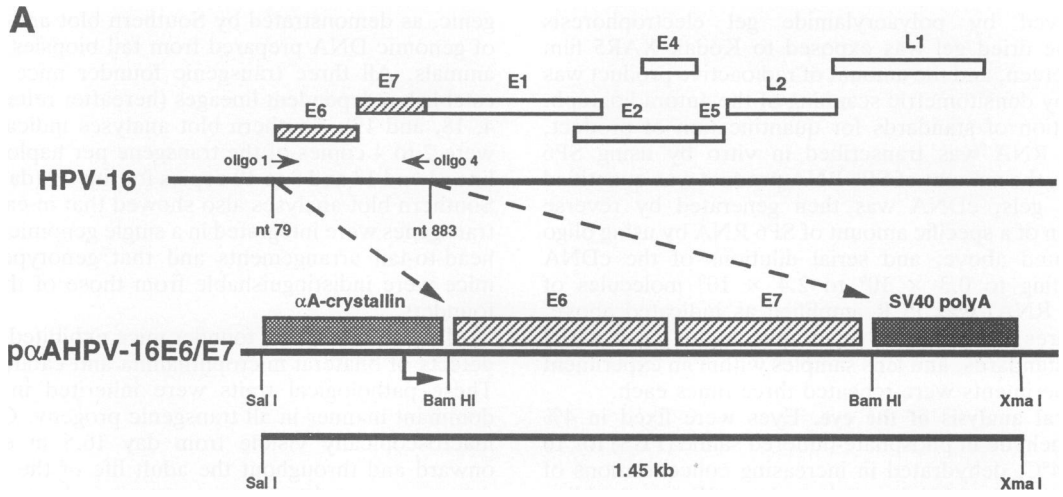
αAHPV-16E6/E7 transgenic mice exhibit overt lens defects. To express E6 and E7 specifically in the ocular lens, the genomic sequences encoding the HPV-16 E6 and E7 open reading frames were amplified by PCR and inserted into the vector αIpA at the unique *Bam*HI restriction site (Fig. 1A). This vector contains the 5′ transcriptional regulatory sequences for the murine αA crystallin gene upstream and the SV40 polyadenylation sequences downstream of the *Bam*HI site. For microinjection, the *Xma*I-*Sal*I fragment containing the αAHPV-16E6/E7/pA fusion was injected into one-cell FVB/N mouse embryos. Three of 27 pups born were transgenic, as demonstrated by Southern blot and PCR analyses of genomic DNA prepared from tail biopsies of 3-week-old animals. All three transgenic founder mice were bred to establish independent lineages (hereafter referred to as lines 4, 18, and 19). Southern blot analyses indicated that there were 2 to 4 copies of the transgene per haploid genome for lines 4 and 18 and 8 to 10 copies for line 19 (data not shown). Southern blot analyses also showed that in each lineage the transgenes were integrated in a single genomic site in tandem head-to-tail arrangements and that genotypes of progeny mice were indistinguishable from those of their respective founders.

All three transgenic founder mice exhibited the overt lens defects of bilateral microphthalmia and cataracts (Fig. 1B). These pathological traits were inherited in a genetically dominant manner in all transgenic progeny. Cataracts were macroscopically visible from day 16.5 in embryogenesis onward and throughout the adult life of the animal. These defects appeared to be mouse strain independent; microphthalmia and cataractous phenotypes were stably inherited when the αAHPV-16E6/E7 transgene (lines 4 and 19) was crossed onto the mouse strain C57BL/6N genetic background. Within a transgenic line the pathological traits appeared consistent; between transgenic lines the severity of the defects differed, with line 19 exhibiting the most severe phenotype in that they had the largest cataracts.

Lens tumors developed in greater than 40% of line 19 αAHPV-16E6/E7 transgenic mice (Fig. 1B). By 6 months of age, 13 of 28 αAHPV-16E6/E7 line 19 homozygous mice developed lens tumors. Lens tumors also developed in line 19 heterozygotes, although they didn't appear until 12 months of age (in 6 of 13 mice that have reached this age). Tumor formation occurred both unilaterally and bilaterally; a total of 21 lens tumors have been identified to date. In contrast, no lens tumors have so far been seen in line 4 and line 18 mice (in 40 to 50 animals exceeding 1 year of age, including homozygotes in line 4).

Expression of E6 and E7 in transgenic mouse tissues. We predicted that both E6- and E7-specific mRNAs would be transcribed from the αA crystallin promoter in these transgenic mice through differential splicing, similar to their expression from the endogenous HPV-16 P_{97} promoter (54). To determine if the observed lens phenotype was correlated with expression of E6 and/or E7 mRNAs, RNA isolated from neonatal and adult lenses was subjected to RNA PCR analysis and then to Southern hybridization to an HPV-16 E6- and E7-specific DNA probe. The strategy and predicted PCR products are outlined in Fig. 2A. Using oligonucleotide 2 for reverse transcription and oligonucleotides 2 and αP1 for subsequent PCR amplification, three amplification products would be predicted; a 523-bp product derived from the unspliced E6-specific transcript, and 348- and 233-bp products derived from the E6*^E7 and E6**^E7 spliced transcripts, respectively. The Southern blot in Fig. 2B indicates that all three predicted amplification products were generated from lens RNA from both neonates and adults in all three transgenic lineages. As occurs in HPV-16-positive human cervical neoplasms, the level of the E6**^E7 transcripts was low in the lens tissue. Generation of these products was sensitive to pretreatment with RNase A and resistant to pretreatment with DNase I, indicating that they arose from mRNA and not contaminating genomic DNA. These transgene specific products were absent in nontransgenic lens RNA samples. Thus, both E6- and E7-specific transcripts are present in the lens at both neonatal and adult stages.

To compare the E6- and E7-specific RNA levels between



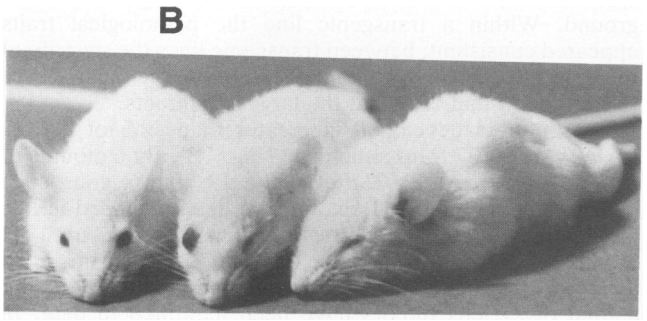


FIG. 1. Construction of the αAHPV-16E6/E7 transgene and phenotypes in transgenic mice. (A) Construction of the αAHPV-16E6/E7 transgene. The top line shows a linear diagram of the HPV-16 genome. Boxes indicate the positions of the early and late open reading frames along the map. The E6 and E7 open reading frames (striped boxes located between nucleotide 79 and 883 on the genome) were amplified by PCR with oligonucleotides 1 and 4, as indicated in Materials and Methods, and cloned between the murine αA crystallin promoter and SV40 polyadenylation sequences at the unique *Bam*HI site (bottom line) to create the plasmid pαAHPV-16E6/E7. The arrow within the αA crystallin promoter represents the position of transcription initiation and direction of transcription. The entire transcriptional unit of 1.45 kb was isolated from the bacterial plasmid by digestion with the restriction endonucleases *Sal*I and *Xma*I, purified and microinjected as described in Materials and Methods. (B) Phenotypes of αAHPV-16E6/E7 adult transgenic mice. Shown are a nontransgenic littermate (left), a line 19 αAHPV-16E6/E7 transgenic mouse with a lens tumor in one eye and the microphthalmia and cataract in the other eye (middle), and a line 19 αAHPV-16E6/E7 transgenic mouse with two microphthalmic, cataractous eyes (right).

different lineages, quantitative RNA PCR was performed on neonatal lens samples (Fig. 2C) by using oligonucleotides 3 and 4 which amplify an RNA segment that is colinear for E6, E6*^E7 and E6**^E7 transcripts (Fig. 2A). For these experiments, one PCR oligonucleotide primer was radiolabeled with [γ-^{32}P]ATP by using T4 polynucleotide kinase, and serial dilutions of reverse-transcribed RNA were amplified by PCR for 25 cycles. The radioactive PCR products from these experimental reaction mixtures and from standard reaction mixtures containing serial dilutions of reverse-transcribed E6 and E7 RNA were resolved by PAGE and quantified by autoradiography and densitometric scanning. Preliminary experiments were performed to establish conditions that yielded a linear relationship between levels of input RNA and amount of product. On the basis of this analysis, transgene RNA levels were reproducibly (three separate experiments, performed in quadruplicate) six to eight times higher in line 19 neonatal lenses than in line 4 and 18 lenses.

To examine the tissue specificity of transgene expression, qualitative RNA PCR analysis was performed on several neonatal tissues from all three transgenic lines (Fig. 2D). The expression pattern was highly specific in line 18, in which E6 and E7 expression was detected in the lens but not in liver, brain tissue, intestine, tail, or the remainder of the eye, although urokinase was detected. Expression of E6 and E7 in line 4 transgenic mice was somewhat less specific, as transcripts were detected not only in the lens but also in the remainder of the eye (the latter could be due to the presence of residual lenticular tissue left during tissue preparation) and in the brain. We cannot rule out the possible expression of transgenes in line 4 and 18 tails, as the positive control RNA (urokinase) was not detected in those tissues in this particular experiment. Expression of E6 and E7 in line 19 transgenic mice was the least specific, as transcripts were detected in all tissues examined except liver. Ectopic expression of transgenes under the transcriptional control of the αA crystallin promoter has been reported previously; for example, the SV40 tumor antigens are expressed in extralenticular sites in certain αASV40TAG transgenic mouse lines, and this expression is associated with the development of nonlenticular tumors in adult mice (35). To date, no gross pathologic, phenotypic, or behavioral abnormalities have been noted in association with expression in the brain of line 4 or 19 mice or intestine of line 19 mice. Abnormalities on the skin and ears of line 19 adults have been noted (28), and their relationship to E6 and E7 expression is being investigated.

Impaired differentiation and hyperproliferation of αAHPV-16E6/E7 transgenic lens cells in vivo. The lenses from αAHPV-16E6/E7 transgenic mice were analyzed histologically to assess the cause of the cataractous, microphthalmic phenotype. The normal developmental program of the lens proceeds

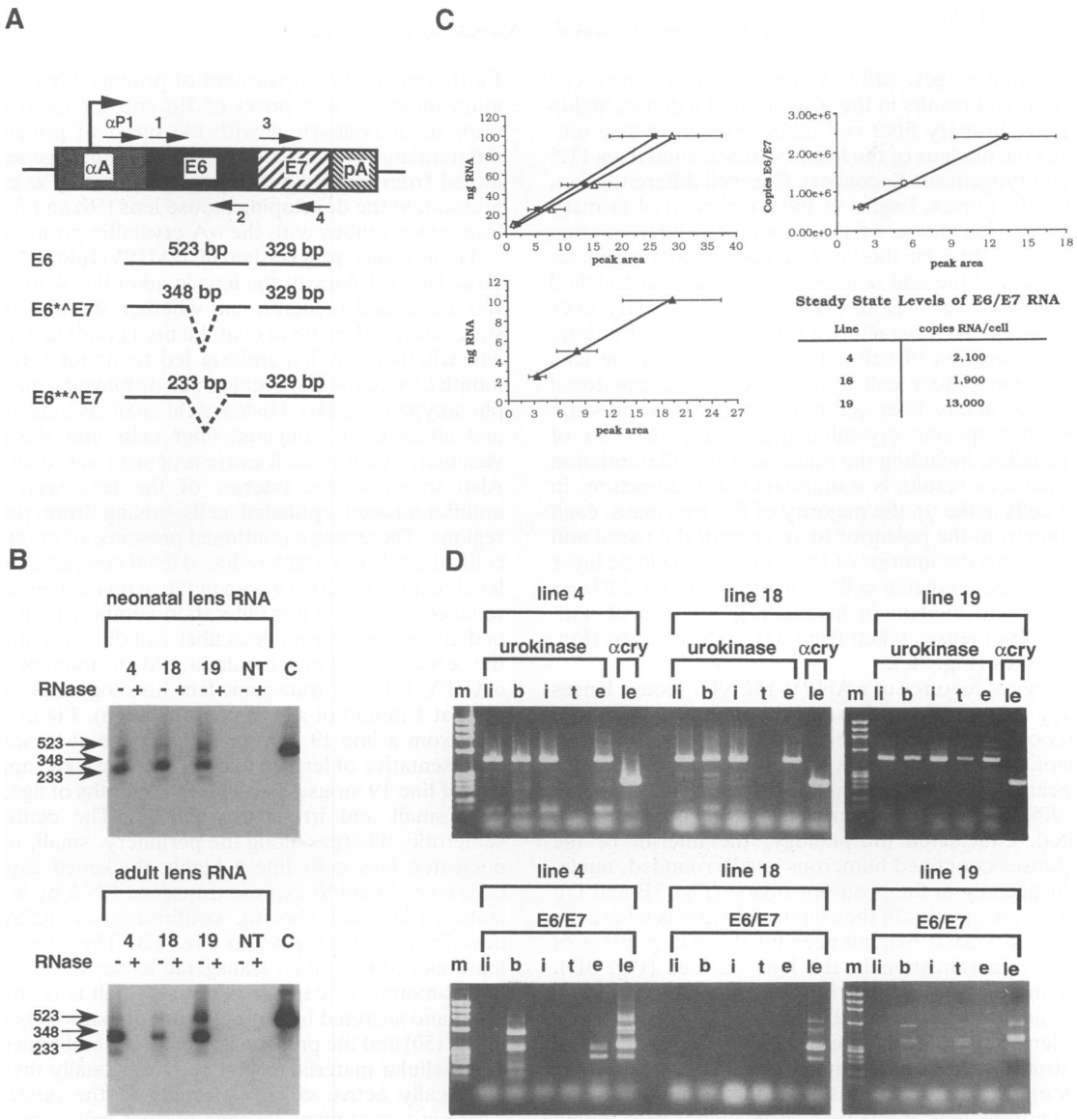


FIG. 2. Expression of HPV-16 E6 and E7 in transgenic mouse tissues. (A) Predicted products of RNA PCR analysis for E6 and E7. The top line shows a schematic representation of the transgene. αA, murine αA crystallin promoter unit; E6 and E7, HPV-16 E6 and E7, respectively, open reading frames; pA, SV40 polyadenylation sequences. The arrows labeled αP1, 1, 2, 3, and 4 indicate the annealing positions of the oligonucleotide primers used for RNA PCR along the transgene. The large directed arrow indicates the transcription initiation site of the transgene within the αA crystallin promoter. The lower portion of the panel represents the predicted sizes for PCR products resulting from E6, E6*^E7, and E6**^E7 messages when the pairs of oligonucleotide primers used were either oligo αP1-oligo 2 or oligo 3-oligo 4. Dotted lines represent splicing within the E6*^E7 and E6**^E7 transcripts. (B) Southern blot hybridization of qualitative RNA PCR analysis of E6 and E7 expression in neonatal and adult αAHPV-16E6/E7 lenses. RNAs isolated from lenses of neonates and adults were treated with RNase-free DNase (−), and half of the sample was then treated with RNase A (+). RNAs were subsequently reverse transcribed with oligo 2 and finally amplified by PCR with oligonucleotides αP1 and oligo 2. The PCR products were separated on 2% agarose gels, transferred to nitrocellulose, and hybridized to [α-^{32}P]-labeled E6 and E7 DNA. The left hand margin indicates the sizes of the hybridizing products. The gel in the top panel suffers from a slight frown which causes misalignment of the bands present in the lane with the arrows to the left. C, positive control for PCR, the pαAHPV-16E6/E7 plasmid; NT, nontransgenic lens; 4, line 4 transgenic lens; 18, line 18 transgenic lens; 19, line 19 transgenic lens. (C) Quantitative RNA PCR analysis of E6 and E7 in lens tissue. Amount of radioactive product obtained from PCR performed (as described in Materials and Methods, performed in quadruplicate, with error bars shown) with stated amounts of input RNA (y axis) is plotted as the integrated peak areas (x axis, obtained by densitometric scanning of autoradiographs). Symbols: ▲, line 19 total cellular RNA; ■, line 4 total cellular RNA; △, line 18 total cellular RNA; ○, T7 polymerase in vitro-transcribed E7 RNA (stock concentration of this E7 RNA was determined by agarose gel electrophoresis with RNA standards). Calculation of the number of copies of the viral RNAs per cell is given in the table and was derived from the following equation: 4×10^{-11} g of RNA per cell, assuming that one cell = 4×10^{-9} g and that RNA = 1% of cell mass. The relative difference in the number of copies of viral RNA per cell between line 19 and either line 4 or line 18 (six to eightfold in this experiment) varied by less than 20% in repeat experiments. (D) Qualitative RNA PCR analysis of E6 and E7 expression in various tissues from neonatal transgenic mice. RNAs isolated from the indicated tissues were subjected to RNA PCR, as described, by using oligonucleotide primers specific for mouse urokinase or mouse αA crystallin, as controls, and HPV-16 E6 and E7. For analysis of E6 and E7, oligo 2 was used for reverse transcription, and oligonucleotides αP1 and 2 were used for PCR amplification. PCR products were electrophoresed on 2% agarose gels and stained with ethidium bromide. The sizes of the products were as follows: urokinase, 469 bp; αA crystallin, 219 bp; and E6 and E7, 523, 348, and 233 bp. m, ϕX174 *Hae*III digest (1353, 1078, 872, 603, 310, 271/281, 234, 194, 118, and 72 bp, top to bottom); li, liver; b, brain; i, intestine; t, tail; e, eye without lens; le, lens.

in two sequential stages, primary and secondary fiber cell differentiation, and results in the formation of a dense, highly ordered tissue. Primary fiber cell differentiation, which ultimately forms the nucleus of the lens, occurs around days 11.5 to 13.5 of embryogenesis. Secondary fiber cell differentiation, which forms the cortex, begins at the conclusion of primary differentiation. This phase of lens fiber cell differentiation proceeds rapidly through the later stages of embryogenesis and early postnatal life and continues, albeit at a diminished rate, as the animal ages. Both primary and secondary fiber cell differentiation are characterized by the onset of mitotic senescence, elongation of cells (in the posterior of the lens vesicle for primary fiber cell differentiation and equatorial epithelia for secondary fiber cell differentiation), expression of differentiation-specific crystallin genes, and the loss of cellular organelles, including the nucleus. This differentiation process in the lens results is a multilayered architecture, in which fiber cells make up the majority of the lens mass, each cell stretching from the posterior to anterior of the tissue and tightly packed into the interior of the lens, with a single layer of undifferentiated epithelial cells along the anterior surface. These features are evident in histological sections of nontransgenic FVB/N lenses taken from day 16.5 embryos (Fig. 3A) and neonates (Fig. 3C).

The histological features of αAHPV-16E6/E7 mouse lenses are strikingly different from those of the nontransgenic lens. This difference was evident in both day 16.5 embryos (Fig. 3B) and neonates (Fig. 3D). Grossly, the transgenic mouse lenses appeared disorganized and dysplastic. In place of terminally differentiated lens fiber cells that are characterized by elongated, enucleated morphology, the interior of the transgenic lenses contained numerous small, rounded, nucleated cells, especially in the posterior region (Fig. 3B and D). Also present were cavities in the equatorial regions where the lumen of the lens vesicle had not been filled and large areas of sclerosis and mineralization in the lens nucleus (Fig. 3D). Higher-magnification views of the posterior (Fig. 3E) and equatorial and anterior (Fig. 3F) regions of the neonatal transgenic lens illustrate the presence of mitotically active cells, the absence of cellular elongation, and a thickening of the anterior epithelium. In contrast, the only region of mitotic activity in a normal lens at this time in development is within a portion (proliferation compartment) of the equatorial epithelium (37). Abnormal presence of nucleated cells within the body of the lens and the absence of cell elongation was also evident in the day 16.5 embryonic transgenic lens (Fig. 3B). By immunohistochemical analyses, the differentiation-specific β and γ crystallins were found expressed in the morphologically undifferentiated lens cells within these transgenic lenses (data not shown). This indicates that there was an uncoupling in the regulation of crystallin gene expression from morphological differentiation, as noted previously for transgenic mice expressing other DNA tumor virus oncogenes, SV40 T antigens (35) and polyoma large T antigen (17). The histology of line 19 neonatal lens was similar to that seen for line 4 and line 18, except that the hyperplasia was more extensive, as evidenced by a higher apparent mitotic index (data not shown).

These histological features of the αAHPV-16E6/E7 lens indicate that the transgenic lens cells have failed to undergo the normal program of primary and secondary fiber cell differentiation. In place of terminal differentiation there is evidence for abnormal proliferation. These abnormalities in the regulation of lens cell proliferation and in morphological differentiation are likely to be responsible for the cataractous appearance of the embryonic and neonatal transgenic lens. Furthermore, the impairment of primary fiber cell differentiation indicates that onset of E6 and E7 expression occurs prior to or concurrent with the onset of primary fiber cell differentiation (around day 11.5 in embryogenesis), as predicted from studies describing the onset of α crystallin expression in the developing mouse lens (50) and from previous transgenic studies with the αA crystallin promoter (42, 43).

Tumorigenic progression of αAHPV-16E6/E7 lens cells in vivo. The histology of the lens in adult line 4 and line 19 mice was examined to determine whether the hyperproliferative state observed in the neonatal lenses continued into adult life and whether this hyperplasia led to tumor formation. At 1 month of age, the transgenic mice displayed a microphthalmic phenotype (Fig. 1B). Histological analysis indicated a continued absence of elongated fiber cells and the presence of vacuolated cells as well as areas of sclerosis (data not shown). Also found in the interior of the lens were pockets of undifferentiated epithelial cells arising from the equatorial regions. There was a continued presence of mitotically active cells, though at a much reduced level compared with the high level of mitotic activity seen in the neonatal lens sections. The reduced level of cell proliferation combined with the continued absence of normal lens fiber cell differentiation results in the observed microphthalmia. At 6 months of age the αAHPV-16E6/E7 transgenic lens histology was similar to that seen at 1 month of age (data not shown). Figure 4A shows a lens from a line 19 homozygous mouse at 8 months of age, representative of lens histology from line 4 and approximately half of line 19 mouse eyes at 6 to 8 months of age. The lens is very small and irregularly shaped. The center is highly schlerotic, whereas along the periphery, small, rounded, and nucleated lens cells line a highly thickened capsule. These cells were found to express transgene RNA by in situ hybridization (data not shown), confirming the RNA PCR data described in the legend to Fig. 2B. The most pronounced features of these older transgenic lenses are the presence of large amounts of capsule material which is normally synthesized and secreted by lens cells at comparatively much lower levels (50) and the presence of lens cells embedded within this extracellular material (Fig. 4B). Occasionally there are found mitotically active cells, especially in the posterior region. There is a continued absence of fiber cell elongation. These unusual histological features demonstrate that the cells in adult αAHPV-16E6/E7 transgenic lenses retain their proliferative capacity, but at much reduced frequency compared with that observed in embryos and neonates, and continue to be impaired in their abilities to differentiate.

As indicated above, lens tumors developed in greater than 40% of line 19 mice. Shown in Fig. 4D is a histological section of an eye tumor from an 8-month-old line 19 homozygous mouse, representative of lens tumors seen to date in this line. This section reveals a large mass of proliferating, neoplastic lens cells which has grown outside the broken lens capsule and completely filled the eye. Histologically, the tumor cells appear moderately differentiated and were verified to be lenticular in origin on the basis of α crystallin-positive immunofluorescence and immunoblot analyses (data not shown). Some cells are multinucleated. There is thickening of the corneal stroma, and in some places the corneal epithelium appears broken, with tumor cells found outside the cornea proper. Neutrophils are present in the corneal stroma as well as in the tumor mass, consistent with there being an inflammatory response. The lens tumor is locally invasive, destroying internal ocular structures such as retina, iris, and ciliary muscle and bodies and breaking into the outermost ocular structures, the cornea at the anterior (Fig. 4E), and the sclera

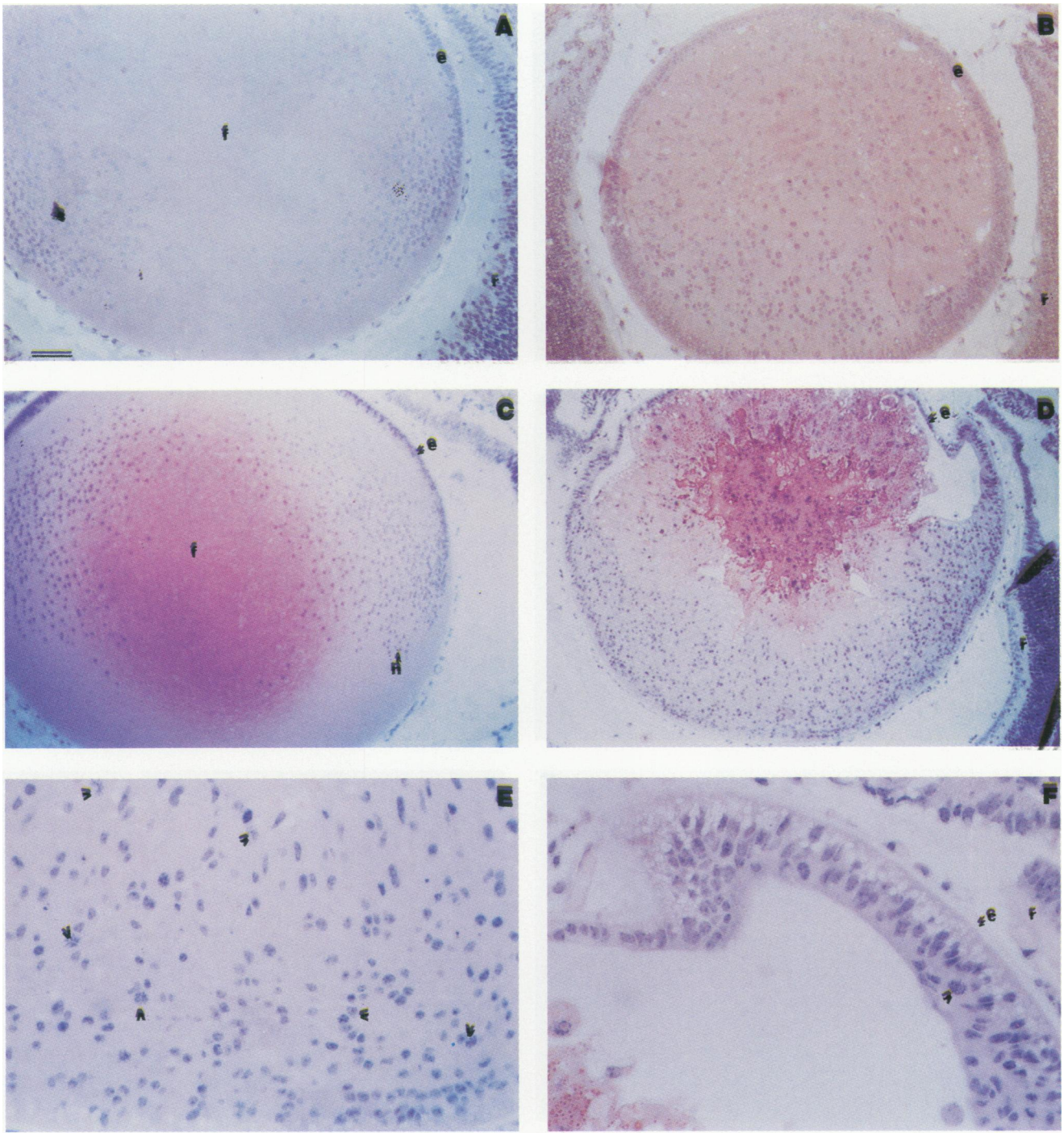


FIG. 3. Histological analysis of lens development in FVB/N nontransgenic versus αAHPV-16E6/E7 transgenic lenses. All eyes were embedded in methacrylate, sectioned, and stained with hematoxylin and eosin. Representative sections from FVB/N day 16.5 embryos (A) and neonates (C) and from line 4 αAHPV-16E6/E7 day 16.5 embryos (B) and neonates (D) are shown. (E and F) higher magnification of the posterior region (E) and epithelial region (F) of the αAHPV-16E/E7 lens shown in panel D. Bar, 5.5 μm for panels A and B, 11 μm for panels C and D, and 3 μm for panels E and F. c, lens capsule; e, epithelial cell; f, fiber cell; n, cell nucleus; r, retina; arrowhead, mitosis. In all panels, the anterior of the lens is oriented at the top of the panel.

at the posterior (Fig. 4F). Whereas the normal lens is an avascular tissue, the tumor mass is infiltrated by numerous blood vessels, indicative of angiogenesis (Fig. 4D to F). Blood vessels are also evident in the normally avascular cornea. In total, the histological features of this eye indicate the presence of a locally invasive, highly vascularized, malignant lens tumor. Similar histological properties were evident in other lens tumors. On the basis of RNA PCR analysis, cell lines

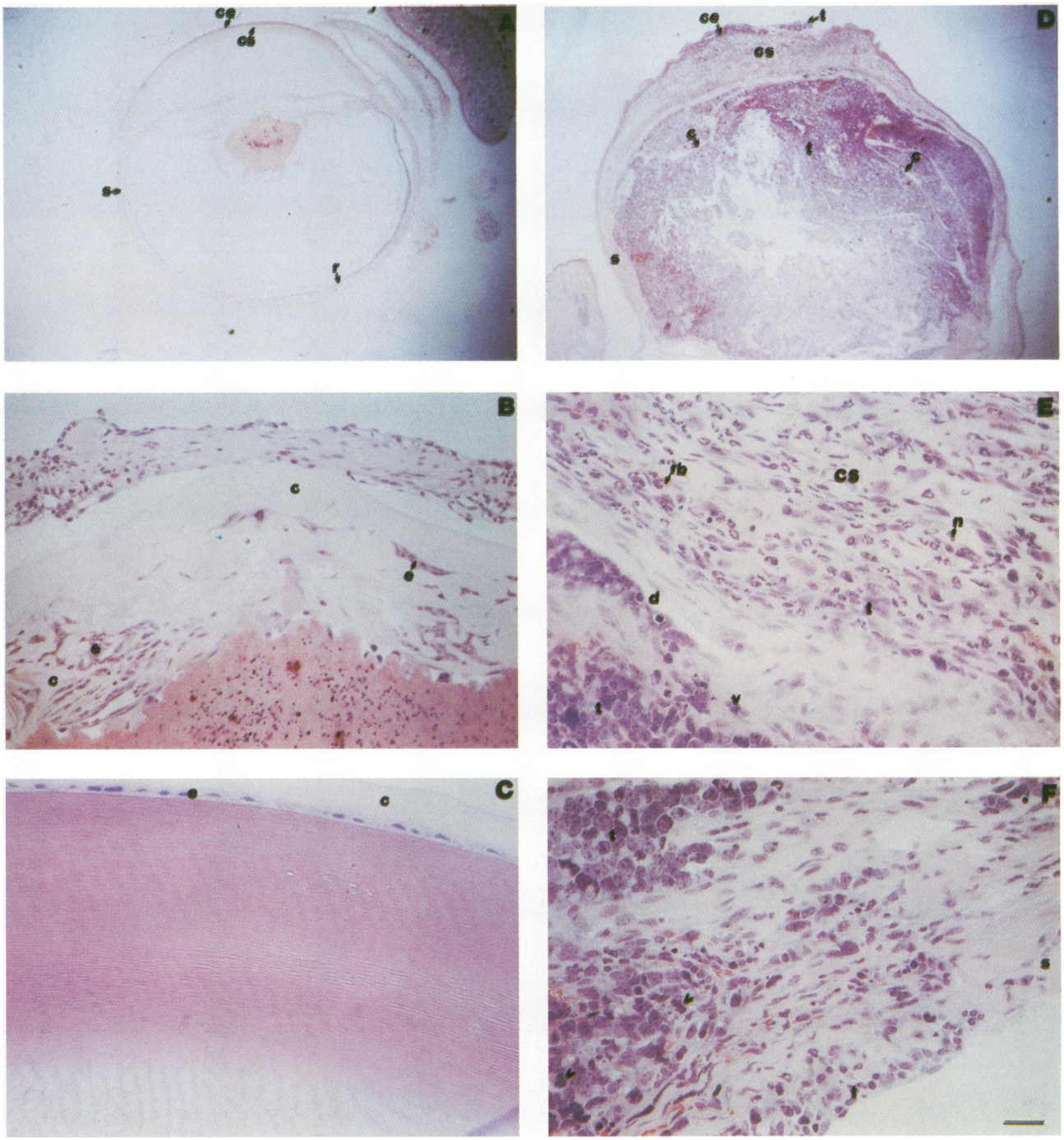


FIG. 4. Histological analysis of adult lenses in line 19 αAHPV-16E6/E7 transgenic mice. All eyes were embedded in methacrylate, sectioned, and stained with hematoxylin and eosin. Representative sections are shown from an eye without a tumor from an 8-month-old line 19 homozygote (A), the anterior region of the lens shown in panel A (B), the anterior region of a lens from a 6-month-old nontransgenic FVB/N mouse (C); eye containing a tumor from an 8-month-old line 19 homozygote (D); and higher magnification of the anterior (E) and posterior (F) regions of the tumor. Bar, 44 μm for panels A and D; and 3 μm for panels B, C, E, and F. c, lens capsule; ce, corneal epithelium; cs, corneal stroma; d, Descemets membrane; e, epithelial cells; n, neutrophils; r, retina; rb, erythrocytes; s, sclera; t, tumor cells; arrowhead, mitosis. In all panels, the anterior of the eye is oriented at the top of the panel.

derived from tumor tissue expressed the E6 and E7 transgenes (data not shown). Tumor tissue and the derived cell lines were tumorigenic upon transplantation into syngeneic neonates (data not shown).

Tumorigenic progression of αAHPV-16E6/E7 lens cells in vitro. Hyperplasia is thought to be a prerequisite for tumorigenic progression, allowing for the accumulation of necessary secondary genetic changes that provide the cell a selective growth advantage. In this study, there appeared to be a correlation between high levels of E6 and E7 mRNA,

high proliferative potential of the mouse lens cells (as indicated by the high mitotic index in neonatal lenses), and tumor incidence in line 19 compared with lower values in all three categories for lines 4 and 18. We also observed a decreased presence of mitotically active lens cells in older animals in all three lines (representative data seen in Fig. 4B), despite a continued expression of the E6 and E7 genes (Fig. 2B). Prior studies had suggested that the adult ocular environment has the capacity to suppress lens cell proliferation (17). Furthermore, it has been demonstrated previously that HPV-18 E6 and E7 immortalized cells can become tumorigenic spontaneously with passage in tissue culture (22, 46). We were interested, therefore, in determining whether the lens cells from line 4 mice, which did not form tumors spontaneously in vivo, could become tumorigenic when permitted to proliferate outside the potentially restrictive environment of the eye.

Lens epithelial cell lines were established from neonatal heterozygous line 4 transgenic mice. Lens cells from nontransgenic neonates have a very limited lifetime in culture, senescing after approximately 20 generations or four to five passages. In contrast, the line 4 αAHPV-16E6/E7 lens cells could be maintained in continuous culture for greater than 8 months, or 40 passages, demonstrating that these cells behave as if they are immortalized. To look for any evidence of spontaneous progression, cellular transformation was measured by soft agar assays (Table 1). Cells from passage 3, nontransgenic FVB/N primary lens cells, and NIH 3T3 cells did not form colonies in soft agar (Table 1). After 14 passages in tissue culture, αAHPV-16E6/E7 lens cells had very low colony-forming efficiency when plated in soft agar. After further passaging, αAHPV-16E6/E7 lens cells displayed higher colony-forming efficiency when plated in soft agar. Colonies were isolated from the soft agar plates of P14 αAHPV-16E6/E7 cells, cells were expanded, and their ability to grow in soft agar was verified (agar clones 10, 12, 14, 16, and 18 [Table 1]). Thus, by these criteria, transformation occurred spontaneously with continued cell proliferation in tissue culture.

To assess whether the above transformation property correlated with tumorigenicity, αAHPV-16E6/E7 lens cells from various passages and from isolated soft agar clones were injected into neonatal syngeneic mice (Table 2). Cells from early (passage 2, 4, and 6) αAHPV-16E6/E7 lens cultures had no capacity to form tumors in mice, while high-passage (passage 24 and 36) cells that possessed the capacity to form soft agar colonies efficiently were also efficient at producing tumors, with palpable tumors arising within 3 weeks of injection. Cells from passage 15 and 17 that showed very low levels of colony formation in soft agar had a limited capacity to form tumors in mice, with reduced efficiency (2 of 12 mice injected) and longer latency (6 versus 3 weeks). Clones isolated from soft agar yielded tumors in proportion to their efficiency of cloning in agar; specifically, agar clone 10, which had a relatively low level of colony growth compared with the other clones, was inefficient at forming tumors. Tumors were directly transplantable (Table 2), and cells isolated from primary tumors, when cultured and then reinjected into mice, were also tumorigenic (data not shown). Thus, whereas line 4 lenses did not develop tumors spontaneously in vivo, line 4 lens cell populations could acquire the ability to form colonies efficiently in soft agar and to form tumors in syngeneic animals when permitted to proliferate outside the lens environment. These results are consistent with the proposal that the lens environment is restrictive to lens cell proliferation and that this restriction indirectly inhibits transformation.

TABLE 1. Acquired capacity for colony growth in soft agar[a]

Cell line	No. of colonies in 0.35% Noble agar
αAHPV-16E6/E7, line 4-1	
P3	0
P14	71
P23	482
P30	880
P36	896
Line 4-1, P14	
Agar colony 10	66
Agar colony 14	752
Agar colony 16	688
Agar colony 18	1,152
Nontransgenic FVB/N lens cells	0
NIH 3T3	0

[a] The αAHPV-16E6/E7 line 4-1 cells from the passages (P) indicated above were seeded at 10^5 cells in 0.35% Noble agar as described in the text. After 4 weeks in culture, plates were scored for the number of anchorage-independent colonies greater than 0.1 mm in diameter. Individual soft agar colonies derived from passage 14 cells were isolated, expanded as clonal populations, and then retested for growth in soft agar.

DISCUSSION

In this transgenic mouse study, we demonstrate that the primary in vivo effects of the E6 and E7 genes on an epithelial cell type is the induction of cell proliferation and impairment of cell differentiation. Similar histological features are found in HPV-positive cervical dysplasias, specifically the hyperproliferation of basal epithelial cells and the impairment of epithelial cell differentiation. The primary in

TABLE 2. Tumorigenicity of passaged αAHPV-16E6/E7 line 4-1 cells in syngeneic mice

Cell source[a]	No. of mice with palpable tumors[b]/no. of mice injected	
	Expt 1	Expt 2
αAHPV-16E6/E7, line 4-1		
P2/4/6	0/6	0/6
P15/17	2/6 (6)	0/6
P24		6/6 (3)
P36	6/6 (3)	5/5 (3)
Line 4-1, P14		
Agar colony 10	0/6	1/2 (4)
Agar colony 16	4/4 (3)	3/3 (3)
Agar colony 18	6/6 (3)	4/6 (3)
αAHPV-16E6/E7, line 4-1		
P36 tumor		7/7 (3)
Agar colony 16 tumor		3/4 (3)
Agar colony 18 tumor		3/4 (3)

[a] 10^7 cells injected per 75 μl of Dulbecco's modified Eagle medium or minced tumor injected subcutaneously into 3- to 4-day-old FVB/N or αAHPV-16E6/E7 mice. Mice were monitored for up to 12 weeks for tumor formation. See Table 1, footnote *a*, for descriptions of cell lines and abbreviations.

[b] With the exception of a few mice, animals were euthanized when tumors were greater than 1 cm in diameter. Numbers in parentheses indicate time in weeks when palpable tumors were found.

vivo effects of HPV-16 E6 and E7 corresponded in tissue culture with the immortalization of lens cells, similar to that seen in human foreskin keratinocytes in tissue cultures harboring transfected HPV-16 E6 and E7 genes. In addition, we demonstrate the frequent occurrence of tumors in the line of αAHPV-16E6/E7 mice expressing the highest levels of E6 and E7 RNA and the spontaneous progression of transgenic lens cells passaged in tissue culture, consistent with the hypothesized role of the HPV-16 E6 and E7 genes in the development of cervical carcinoma. Thus, our studies implicate the E6 and E7 genes as being important viral agents in the pathology of HPV infection and associated cancer. These transgenic mice should provide a useful model system for exploring further the role of these viral oncogenes in human cancer and in the use of these oncogenes as probes into the mechanisms that control cell growth and differentiation during normal development.

Tumorigenicity of HPV-16 E6 and E7. Tumor formation in the lens was seen in greater than 40% of older line 19 transgenic mice. This line of mice had the highest levels of E6 and E7 RNA transcripts in neonates (Fig. 2C). Together, these observations indicate that the incidence of progression correlates with high levels of viral oncogene expression and are consistent with the hypothesis that high-level expression of HPV-16 E6 and E7 in cervical carcinoma plays an important role in the etiology of this cancer (63).

While tumors in ocular structures such as the retina are known to occur, spontaneous lens tumors have not been reported (51). This finding leads to the hypothesis that the intraocular microenvironment of the lens suppresses spontaneous lens tumorigenesis. The lack of tumorigenesis may be related in part to an active restriction of adult lens cell growth. Our evaluation of line 4 lens cell populations in vivo and in tissue culture is consistent with this hypothesis. In vivo, line 4 lens cells exhibited high proliferative capacity in neonates, but not in adults. However, when placed in cell culture, these transgenic lens cells were immortal and displayed the potential to progress, as demonstrated by the acquired capacity to grow as colonies in soft agar (Table 1) and to form aggressively growing tumors when injected into syngeneic mice (Table 2). We conclude that the absence of spontaneously occurring tumors in line 4 (and in line 18) mice results at least in part from the inability of line 4 lens cells to overcome negative growth regulatory signals present in the adult in vivo lens environment rather than from an inherent inability for these lens cells to proliferate or progress per se. Consistent with this conclusion is the prior observation that lens cells which are phenotypically immortalized by polyoma large T antigen are nevertheless responsive to the growth inhibitory effects of bFGF and insulin (17), growth factors that are believed to be regulators of lens cell differentiation (3, 5). That line 19 cells possess the capacity to progress in vivo may indicate that sufficient levels of E6 and E7 can overcome the suppression of lens cell proliferation by environmental factors.

The long latency in tumor formation in the line 19 αAHPV-16E6/E7 mice is consistent with the need for the accumulation of additional genetic changes in the lens cell during progression. This late adult onset tumorigenesis is comparable to the temporal appearance of testicular tumors in adult transgenic mice expressing HPV-16 E6 and E7 genes from the mouse mammary tumor virus transcriptional control elements (27), though in the latter study there was no correlation between viral oncogene expression and any premalignant pathology. Similar to the lens tumors described in our study, the testicular tumors appeared at approximately 6 to 8 months of age, were vascularized and locally invasive, but were not metastatic, on the basis of gross examination, at the time the animals were sacrificed. The efficient induction of tumors in αAHPV-16E6/E7 mice and the generation of a series of cell populations in tissue culture that have acquired a tumorigenic potential with cell passage should provide us the means to identify those genetic changes which are most frequently associated with progression, as has been supplied previously by transgenic mice expressing the bovine papillomavirus transforming gene E5 (24, 33).

Comparison of E6 and E7 to other viral oncogenes expressed in the transgenic mouse lens. The lens has been utilized as a target for the expression of several viral oncogenes through the use of the αA crystallin promoter, providing a basis for comparing their in vivo activities. Our data indicate that the HPV-16 E6 and E7 genes display an intermediate potential for altering epithelial cell growth and differentiation and in inducing tumor formation. By far the most active oncogenes tested to date in the lens are the SV40 T antigens. Expression of the SV40 early region in the lens resulted in efficient and rapid onset of tumors apparent in many lines at birth (35). One SV40 transgenic line, αT2, however, displayed latencies of up to 4 months before tumor formation was detected, and this latency correlated with both the time of onset and level of expression of the SV40 transgenes (42). The ocular pathology of the αAHPV-16E6/E7 line 19 mice resembles that observed for the αT2 line. The polyomavirus large T antigen gene (PyLT) did not induce tumor formation in the mouse lens, though these lenses failed to develop normally because of impaired fiber cell differentiation (16). In the αAPyLT neonatal mouse lens, the abundance of proliferating cells was much lower than in the αAHPV-16E6/E7 mouse lens, which in turn was lower than in the αT1 (rapid tumor onset line) mouse lens. Thus, the degree to which the viral oncogenes disrupted normal epithelial cell growth and differentiation correlated with the propensity for tumor formation.

The differences in the biological activities of these viral oncoproteins in the transgenic mouse lines may relate to differences in their biochemical properties, specifically, their capacity to interact with cellular tumor suppressor gene products. The SV40 large T antigen is known to associate with both p53 (31, 34) and Rb (10), the same tumor suppressor gene products targeted by the HPV-16 E6 and E7 proteins (13, 60). In both cases, transgene expression resulted in tumor formation. In contrast, the polyomavirus large T antigen only binds to Rb (12), and its expression in the lens did not correlate with tumorigenesis. A simple interpretation is that the inactivation of p53 may contribute to tumorigenesis in the lens, consistent with the high incidence of p53 mutational inactivation seen in many cancers (32). Other factors, however, may contribute to the differences observed among these transgenic lines, including (i) the expression of small t antigen in SV40 transgenic mice (8, 42), (ii) the timing of expression of the transgene during lens development (42), and (iii) the levels of expression of the transgenes (42; this study).

E6 and E7 as probes for understanding growth control during tissue development. The primary biological effect of E6 and E7 in the transgenic lens was the efficient (100% penetrance in all three lines of transgenic mice) impairment of cell differentiation and induction of proliferation, histological features seen in HPV-16-infected tissue and associated neoplasias. These transgenic studies may therefore provide a good model system to study the mechanism of

action of E6 and/or E7 in vivo. The best-defined biochemical activities of the E6 and E7 proteins are their capacity to bind and inactivate p53 and Rb (13, 47, 53, 60). Since both Rb and p53 genes are expressed in lens cells in vivo (44), it is possible that Rb and/or p53 is the primary target of E6 and E7 action in the lens. This possibility would imply a role for Rb and/or p53 in maintaining the normal regulatory circuits of growth and differentiation of lens cells. However, E6 and E7 possess activities in addition to p53 and Rb binding; E6 exhibits a transcriptional transactivation activity that may or may not be related to its capacity to bind and destabilize p53 protein (30), and E7 is known to bind to other cellular proteins, including p107 (39). Importantly, mutational analyses indicate that the oncogenicity of E7 in tissue culture does not map exclusively to the Rb binding domain (2, 23, 48). Further studies with transgenic mice expressing E6 or E7 individually or mutants of E6 or E7 with altered binding affinities for p53 and Rb will be useful in determining the primary targets of E6 and E7 action in the lens which lead to their effects on cell growth and differentiation. Preliminary data show that when E7 only is expressed in the developing lens, a microphthalmic phenotype results, whereas when E6 only is expressed, there are no obvious effects on the embryonic development of the lens (45). Consistent with these preliminary data are recent studies in which a null mutation of p53 was generated in the mouse germline via gene targeting. These p53-deficient mice develop normally but are predisposed to multiple neoplasms as adults (11), again suggesting that p53 function is dispensible for normal embryonic development.

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